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Corticotropin-Releasing Factor Plays a Permissive Role in Cerebellar Long-Term Depression

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Summary

This study of rat cerebellar slices yielded two lines of evidence indicating that the corticotropin-releasing factor (CRF) found in climbing fibers (CFs) is critical for the induction of long-term depression (LTD) at the parallel fiber (PF) synapses of Purkinje cells (PCs) by their conjunctive activation with either stimulation of CFs or depolarization of PCs. First, LTD induction was effectively blocked by specific CRF receptor antagonists, α-helical CRF-(9–41) (α-h CRF) and astressin; and second, LTD was no longer observed in CF-deprived cerebella but was restored by CRF replenishment. The data obtained in this study suggest that these effects are mediated by protein kinase C (PKC) and not by Ca^{2+} signaling or cyclic GMP (cGMP) production.

Introduction

Corticotropin-releasing factor (CRF), a peptide composed of 41 amino acids, is synthesized in the hypothalamus and regulates the release of adrenocorticotropic hormone from the anterior pituitary (Vale et al., 1981). CRF is present not only in the hypothalamo-pituitary system but also in other brain regions (De Souza et al., 1985, 1987; Sakanaka et al., 1987). CRF receptors have also been shown to be present in the brain (Chang et al., 1993; Potter et al., 1994; Chalmers et al., 1995). CRF is reported to be released spontaneously from brain tissues as well as in response to challenges with KCl or acetylcholine (Calogero et al., 1988; Cratty and Birkle, 1994; Gabr et al., 1994). These data suggest that CRF is released both spontaneously and in an activity-dependent manner. CRF may have neuromodulatory functions such as modulation of synaptic transmission and regulation of ionic currents in various brain regions.

In the cerebellum, CRF is concentrated in climbing fiber (CF) afferents, which originate in the inferior olive of the medulla and supply strong excitatory synapses to Purkinje cells (PCs). The entire course of olivocerebellar CF afferents, from the somata of the olivary neurons to CF terminals, has been shown to be immunoreactive for CRF (Parkovits et al., 1987; Sakanaka et al., 1987; Sawchenko and Swanson, 1989; Cummings et al., 1994a). *CRF* mRNA expression and immunoreactivity for CRF of inferior olivary neurons have been demonstrated to increase following experimental manipulations that enhance the activity of these neurons under sustained optokinetic stimulation in the rabbit (Barmack and Young, 1990; Barmack and Errico, 1993) and harmaline treatment in the rat (Cummings et al., 1994b). On the other hand, *CRF* receptor mRNA, mainly for type 1 receptors, is present in PCs as well as granule cells (Chang et al., 1993; Potter et al., 1994). Electrophysiological investigations reveal that CRF acts directly on PCs; in anesthetized rats, CRF enhances the sensitivity of PCs to glutamate and aspartate and reduces their sensitivity to GABA (Bishop, 1990; Bishop and Kerr, 1992; Bishop and King, 1992). CRF also reduces the spike-induced afterhyperpolarization (AHP) in cultured PCs, presumably due to the closure of Ca^{2+}-activated K^+ channels (Fox and Gruol, 1993). Furthermore, spontaneous and KCl-induced release of CRF from cerebellar slices have recently been reported (G. A. Bishop et al., 1997, Soc. Neurosci., abstract). These results provide evidence for the direct actions of CRF on PCs and suggest that CRF may be released from CFs and modulate PC excitability.

One important and well-studied function of the CF system is the triggering of long-term depression (LTD) of parallel fiber (PF) synapses, the other major excitatory input to PCs (Ito et al., 1982; Ekerot and Kano, 1985; reviewed by Ito, 1989). LTD is induced via a complex series of signal transduction processes involving a number of receptors and messengers and eventually causes a persistent reduction in sensitivity to α-amino-3-hydroxy-5-methyl-4-isozazole-propionate- (AMPA-) selective glutamate receptors of PF synapses (reviewed by Daniel et al., 1998). LTD is a form of synaptic plasticity in the cerebellum that has been proposed as the cellular basis for motor learning, such as for adaptation of the vestibulo-ocular reflex (reviewed by Ito, 1982, 1998; Nagao, 1983) and the conditioned eyeblink response (Thompson and Krupa, 1994). CRF expression in olivary neurons has been shown to increase following sustained optokinetic stimulation (Barmack and Young, 1990; Barmack and Errico, 1993), a condition similar to that inducing adaptive enhancement of optokinetic eye movements (Nagao, 1983), suggesting a role for CRF in mechanisms underlying LTD induction and cerebellar motor learning.

Here, we present two lines of evidence indicating that CRF plays a crucial role in the induction of LTD. The first line of evidence was obtained using CRF antagonists, α-helical CRF-(9–41) (hereafter abbreviated to α-h CRF) (De Souza, 1987) and astressin (Gulyas et al., 1995); the second line of evidence was obtained by experiments on CF-deprived rats. We also examined the effects of CRF and its antagonists on Ca^{2+} signaling in PCs and the activation of protein kinase C (PKC) and cyclic GMP

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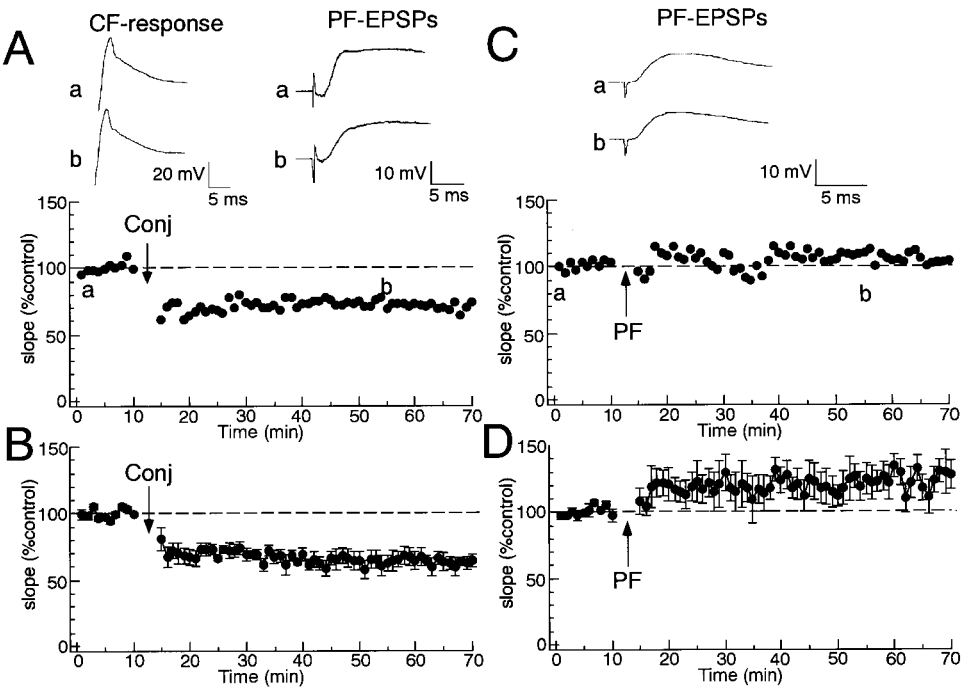

Figure 1. Conjunctive PF and CF Stimulation Induces LTD (LTDcnj)

(A) Conjunctive PF and CF stimulation at 1 Hz for 5 min (300 stimuli) result in LTD of the PF-EPSP initial slopes. Test PF stimulation was repeated at 0.2 Hz. In this and all subsequent figures, the data have been normalized to the mean initial slope value during the control period for the first 10 min. The PF-EPSPs and CF responses shown were recorded 9 min prior to (Aa) and 45 min following (Ab) the initiation of conjunctive stimulation indicated by the arrow. Each trace is an average of five successive responses.

(B) Pooled data (mean ± SEM, n = 14) from the experiments with conjunctive PF and CF stimulation, performed as in (A). Error bars, SEM.

(C) Repetitive PF stimulation alone at 1 Hz for 5 min (300 stimuli) does not produce depression but instead a slight potentiation of PF-EPSP initial slopes. Sample traces were recorded 10 min prior to (Ca) and 45 min following (Cb) the initiation of repetitive PF stimulation indicated by the arrow.

(D) Pooled data (mean ± SEM, n = 9) from experiments with repetitive PF stimulation alone, performed as in (C).

(cGMP) production in cerebellar slices and obtained evidence suggesting that only PKC is involved in the role of CRF in LTD induction.

Results

LTD Induced by Conjunctive PF and CF Stimulation (LTDcnj)

LTD of PF to PC transmission was represented by a decrease in the initial gradient of PF-induced excitatory postsynaptic potentials (PF-EPSPs). LTD was induced by conjunctive PF and CF stimulation at 1 Hz for 5 min (300 stimuli) (Figures 1A and 1B), a protocol that has been shown to be optimally effective for inducing LTD in adult rat cerebellar slices (Karachot et al., 1994). LTD was presumed to have been induced when the decrease in the average initial gradients was larger than 25% both at 10 min and 30 min after conjunctive stimulation; 12 of the 14 PCs tested exhibited LTD. The average decrease in the gradient of the initial rising slope of PF-EPSPs in these 14 PCs was 38.0% ± 4.8% (mean ± SEM at 40 min after the onset of conjunctive stimulation), which is similar to previously reported values (Karachot et al., 1994). Conjunctive stimulation had no effect on complex spikes induced by CF stimulation (Figure 1A, inset). No consistent change in input resistance was associated with LTD (data not shown). Under our recording conditions, repetitive PF stimulation alone at 1 Hz for 5 min did not induce LTD but instead a slight potentiation (Figures 1C and 1D). The average increase of PF-EPSP slope was 23.0% ± 11.0% (n = 9) at 40 min after the onset of PF stimulation. This is consistent with results obtained previously using similar experimental protocols (Sakurai, 1987, 1990; Karachot et al., 1994). Repetitive stimulation of CFs alone at 1 Hz for 5 min caused no change in PF-EPSP slope (0.0% ± 2.0% at 40 min after stimulation, n = 3; data not shown).

α-h CRF and Astressin Antagonize the Effect of CRF on PCs

Two CRF receptor antagonists, α-h CRF and astressin, were used in the present study. α-h CRF is a commonly used CRF antagonist and has been shown to inhibit the effects of CRF with a Ki of 16.7 ± 1.6 nM in brain tissues (De Souza, 1987). Astressin is a novel and more specific antagonist than α-h CRF and has been demonstrated to be 100-fold more potent in an in vitro biochemical analysis (Gulyas et al., 1995) and 17-fold more potent in an in vivo behavioral analysis (Martinez et al., 1997).

We first confirmed that these CRF receptor antagonists antagonized CRF without side effects on the membrane properties or synaptic transmission in PCs. CRF

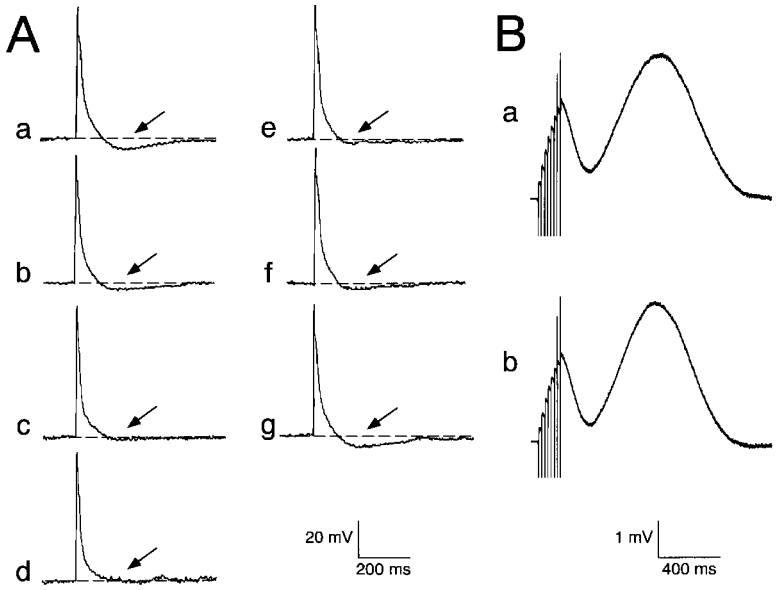

Figure 2. Effects of CRF Antagonist on AHP and mGluR1-Mediated Slow EPSPs

(A) AHPs (arrows) following CF responses as recorded from a PC.

(Aa) Control.

(Ab–Ad) Gradual diminution of AHP, recorded at 5 min (Ab), 10 min (Ac), and 15 min (Ad) after the onset of CRF (1.0 μM) application.

(Ae–Ag) Recovery of AHP in the same PC as in (Aa) through (Ad), recorded at 5 min (Ae), 10 min (Af), and 15 min (Ag) after the onset of addition of α-h CRF (1.0 μM) in the medium subsequent to (Ad).

(B) mGluR1-mediated slow EPSPs evoked in a PC.

(Ba) Repetitive stimulation of PFs (50 Hz, eight pulses) during perfusion with NBQX (10 μM) and AP-5 (30 μM).

(Bb) Similar to (Ba), but under perfusion with α-h CRF (1.0 μM).

has been reported to reduce AHP in PCs that follow spike trains, in a dose-dependent manner (Fox and Gruol, 1993). AHP is presumed to result from activation of both Ca^{2+}-dependent and voltage-gated K^+ conductances (Llinás and Sugimori, 1980). We observed AHP following characteristic complex spikes evoked by stimulation of CFs at 20 s interpulse intervals (Figure 2A, arrows) (Hashimoto and Kano, 1998). Bath application of CRF (1.0 μM) resulted in a substantial reduction in the amplitude of AHP (Figures 2Ab–2Ad, arrow). The peak amplitude of AHP at each time point was estimated relative to the average value obtained for five successive stimuli before CRF application. The AHP amplitude began to decrease within 5 min and reached the level of 23.0% ± 5.0% (n = 6) of the control value at 10–15 min after the onset of CRF application (Figure 2Ad). Consistent with Fox and Gruol's (1993) report, significant decreases in AHP amplitude were induced by 1.0 μM CRF but not 0.1 μM CRF (100.8% ± 0.4%, n = 4). The reduction in AHP amplitude induced by CRF (1.0 μM) was blocked by the subsequent application of α-h CRF (1.0 μM) (Figures 2Ae–2Ag); AHP recovered to 100.0% ± 0.2% (n = 4) compared with the control AHP before CRF application. Similar recovery was also observed after the application of astressin (0.1 μM) (100.0% ± 0.9%, n = 4). AHP was not reduced significantly following application of α-h CRF (1.0 μM) alone for 15 min (97.9% ± 0.3%, n = 6). PF-EPSPs were not affected significantly by application of α-h CRF for 15 min (100.0% ± 2.5%, n = 5) or astressin (101.7% ± 2.2%, n = 5). Neither CF responses, input resistance, nor resting membrane potential were affected by these CRF antagonists.

Neither α-h CRF nor astressin affected fast EPSPs evoked by PF stimulation (see below and Figures 3 and 4), which are mediated by AMPA receptors (Lliano et al., 1991). PF stimulation also evoked slow EPSPs by activation of metabotropic type 1 glutamate receptors (mGluR1s; Batchelor and Garthwaite, 1997; Tempia et al., 1998). We observed that repetitive PF stimulation (50 Hz, eight pulses) evoked slow EPSPs in PCs (average peak size ± SEM, 5.3 ± 0.4 mV, n = 7; Figure 2Ba) following treatment with both 6-nitro-7-sulphamoyl-benzo(f)quinoxaline-2,3-dione (NBQX) (10 μM) to block AMPA receptors and 2-amino-5-phosphonopentanoic acid (AP-5) (30 μM) to block NMDA receptors. These slow EPSPs were not affected by the application of α-h CRF (1.0 μM) (100.3% ± 1.0%, n = 7, relative to the control slow EPSPs; Figure 2Bb).

Blockade of LTDcnj by CRF Receptor Antagonists

To investigate the involvement of CRF in LTD, conjunctive PF and CF stimulation at 1 Hz for 5 min was performed in the presence of α-h CRF (1.0 μM) or astressin (0.1 μM). LTD was not induced in any of the eight PCs tested when conjunctive stimulation was performed 10 min after the commencement of α-h CRF (1.0 μM) application. In three of the eight PCs, conjunctive stimulation resulted in potentiation of the PF-EPSP slope. At 30 min after the onset of conjunctive stimulation, the average value of PF-EPSP slopes in the eight PCs increased to 110.5% ± 12.0% of the control value obtained before the conjunctive stimulation (Figure 3B). On the other hand, in the presence of 0.1 μM astressin, a transient decrease of PF-EPSPs was observed that recovered to the control value within 20 min (Figures 3C and 3D). At 30 min after conjunctive stimulation, the average PF-EPSP slope increased to 105.3% ± 9.0% (n = 11) of the control value obtained before the conjunctive stimulation.

To examine whether CRF is involved in the induction or the maintenance of LTD, conjunctive PF and CF stimulation were performed in the normal perfusion medium, and bath application of α-h CRF (1.0 μM) was started just after the termination of conjunctive stimulation and continued for 15 min (Figure 4A). LTD was induced despite the presence of α-h CRF and was maintained thereafter. The average PF-EPSP slope remained depressed by 30.2% ± 4.6% (n = 6) at 50 min after the

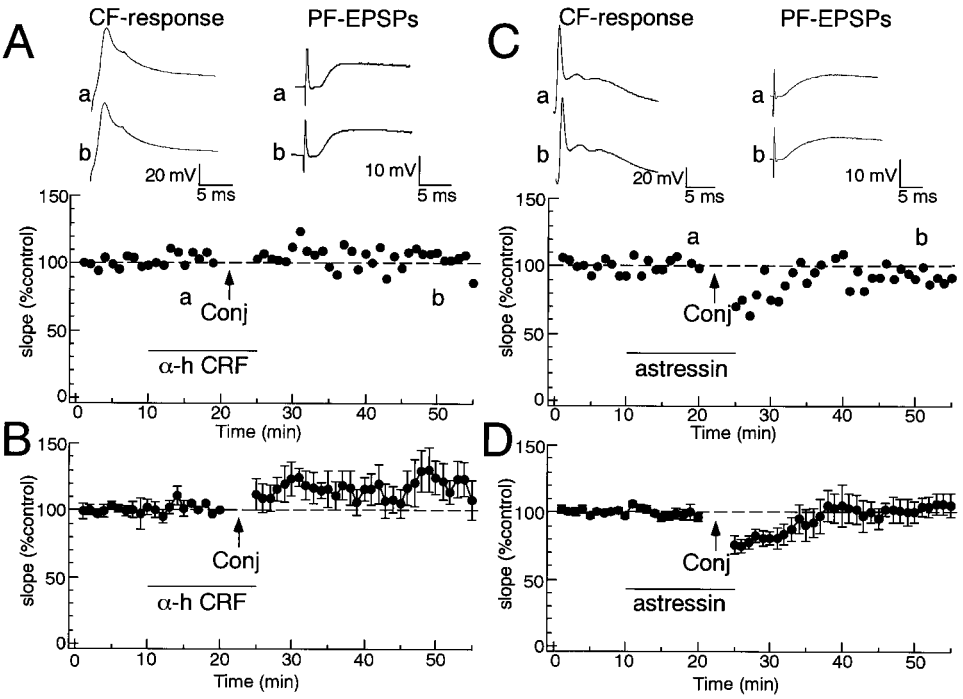

Figure 3. CRF Antagonists α-h CRF and Astressin Block LTDcnj

(A) α-h CRF (1.0 μM) was bath applied during the period indicated by the horizontal bar and prevented LTDcnj. Note that neither PF-EPSP nor CF response was affected by α-h CRF (Aa and Ab).

(B) Pooled data (mean ± SEM, n = 8) from the experiments with conjunctive PF and CF stimulation in the presence of α-h CRF, performed as in (A).

(C) Astressin (0.1 μM) was bath applied during the period indicated by the horizontal bar and prevented LTDcnj. Note that neither PF-EPSP nor CF response was affected by astressin (Ca and Cb).

(D) Pooled data (mean ± SEM, n = 11) from the experiments with conjunctive PF and CF stimulation in the presence of astressin, performed as in (C).

conjunctive stimulation. In another series of experiments, application of α-h CRF was initiated 25 min after the termination of conjunctive stimulation and continued for 15 min (Figure 4B). The antagonist had no effect on the maintenance of LTD (by 32.3% ± 7.1% at 50 min after the conjunctive stimulation, n = 5). These results clearly indicate that CRF is a prerequisite for the induction but not the maintenance of LTD.

LTD Induced by a Combination of PF Stimulation and PC Depolarization (LTDdepo) and Its Inhibition by α-h CRF and Astressin

LTD can also be induced when CF stimulation is replaced with direct depolarization of PCs (Crepel and Jaillard, 1991). A combination of PF stimulation at 1 Hz for 5 min with injection of depolarizing current pulses (200 ms) that are sufficiently large to evoke dendritic Ca^{2+} spikes (Figure 5A, inset) induced LTD of PF-EPSPs (average decrease of 28.5% ± 7.0% at 40 min after the onset of the combined stimulation, n = 7; Figures 5A and 5B). This LTDdepo is similar to LTDcnj (Figures 1A and 1B) in magnitude and time course. LTDdepo was also blocked by α-h CRF (1.0 μM) (Figures 5C and 5D); the transient decrease of PF-EPSPs obtained in response to PF stimulation combined with PC depolarization was followed by a return to control levels within 30 min (Figures 5C and 5D); at 40 min after the combined stimulation, there was no residual decrease of PF-EPSPs (3.0% ± 9.1%, n = 13). Astressin (0.1 μM) also blocked LTDdepo (average decrease at 40 min after the combined stimulation, 5.0% ± 8.9%, n = 4). These results suggest that CRF released by repetitive CF stimulation is not an essential prerequisite for the induction of LTD. Presumably, ambient CRF that is present endogenously in cerebellar slices is sufficient for the induction of LTD (see Discussion).

Abolition of LTD by Chronic Destruction of CFs and Its Restoration by CRF Replenishment

If the CRF contained in and released from CFs is required for the induction of LTD, destruction of the inferior olivary neurons and subsequent degeneration of CF terminals in the cerebellar cortex should result in the abolition of LTD. Hence, we administered 3-acetylpyridine (3AP), harmaline, and niacinamide (3AP-treated) to Wistar rats to selectively destroy the majority of inferior olivary neurons (Llinás et al., 1975). A reduction in PF-induced nitric oxide (NO) production during the 11–16 day period after the 3AP treatment has been reported as a side effect that inhibits LTD induction (Shibuki and Kimura, 1997). However, normal NO production was restored thereafter, such that the NO-dependent cGMP production caused by PF stimulation is indistinguishable from that in normal rats at 50–90 days after 3AP treatment (Okada

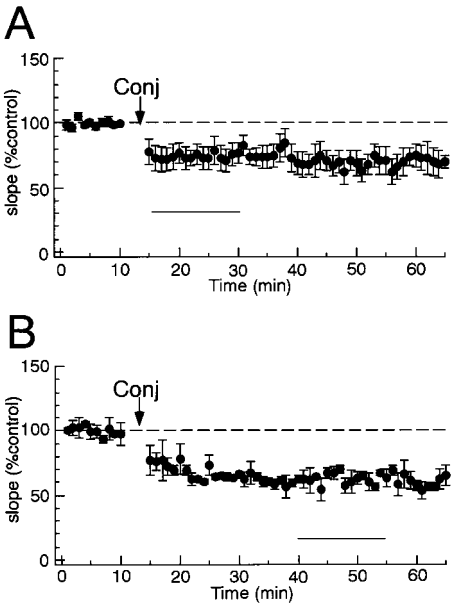

Figure 4. CRF Antagonist Did Not Affect the Late Phase of LTD

(A) Pooled data (mean ± SEM, n = 6) from six experiments with conjunctive PF and CF stimulation (1 Hz, 300 pulses). α-h CRF (1.0 μM) was applied for 15 min immediately after the termination of the conjunctive stimulation, as indicated by a horizontal bar.

(B) Pooled data (n = 5) from five similar experiments in which α-h CRF (1.0 μM) was applied for 15 min, starting 25 min after the termination of the conjunctive stimulation. Error bars, SEM.

and Hartell, 1998). We therefore examined the rats 60–90 days after 3AP treatment. The treated rats developed clear neurological symptoms indicative of cerebellar dysfunction but remained viable. We confirmed histologically in these rats that the inferior olivary neurons disappeared completely, except for a small number of neurons retained at the dorsal cap. In contrast, PC bodies and dendrites were strongly immunoreactive for calbindin D28K in both 3AP-treated and control rats. Homogeneous staining patterns of the molecular layer and PC layer were observed in an overview of coronal sections of the vermis from both groups of animals. We did not observe any absence of staining indicating the loss of PCs in the vermis of 3AP-treated rats. The average number of PCs was 21.38 ± 2.13 (mean ± SD) and 20.93 ± 2.02 per 0.1 mm^2 in 3AP-treated rats (n = 4) and control rats (n = 4), respectively, the difference not being statistically significant ($p > 0.05$, unpaired t test). In situ hybridization experiments indicated that the PCs in 3AP-treated rats expressed the mRNA of *CRF* receptor type 1, similar to untreated rats (M. M., unpublished data). The significant loss of PCs reported by O'Hearn and Molliver (1993) in 3AP-treated rats could be explained by the much higher dose of harmaline used by them (40 mg/kg, body weight single dose, or 25 mg/kg, three doses at 24 hr intervals) compared with our present experiment (15 mg/kg, single dose).

In cerebellar slices obtained from 3AP-treated rats, PF-mediated EPSPs were recorded from PCs. Because some CFs survive the 3AP-treatment (Rossi et al., 1991), care was taken to confirm that only PCs with no CF responses to strong stimuli of the white matter were adopted for the LTD experiments. In such PCs, presumably with no CF innervation, PF stimuli (1 Hz for 5 min) combined with depolarizing current pulses (200 ms) failed to induce LTDdepo in six of eight cells. The average decrease of PF-EPSPs in these eight cells was negligible (2.2% ± 10.0% at 30 min after combined stimulation; Figure 6A). It is unlikely that the inability to induce LTDdepo was due to a reduction of depolarization-induced Ca^{2+} entry, because depolarizing current pulses (200 ms) readily induced dendritic Ca^{2+} spikes (Figure 6A, inset), similar to the observation in control rats. Therefore, the inability to induce LTD was presumably due to the removal of CFs, depriving the cerebellar cortex of endogenous CRF.

LTDdepo was restored when cerebellar slices from the 3AP-treated rats were incubated with extracellular solutions containing 0.1 μM CRF for 3 hr before the recording was commenced (Figure 6B). The average magnitude of the restored LTDdepo was 28.0% ± 10.9% at 30 min after the combined stimulation (n = 7), comparable with the magnitude of the control LTDdepo in normal rats (Figure 5B). No such restoration occurred when the CRF concentration in the perfusates was decreased to 0.01 μM (n = 3). The restoration of LTDdepo by CRF was prevented when 1.0 μM α-h CRF was added to the perfusate containing 0.1 μM CRF (Figure 6C), the average LTD magnitude in this case being 0.6% ± 4.7% (n = 5) at 30 min after LTDdepo-inducing stimulation. This indicates that the rescue of LTD in CF-deprived PCs by CRF is mediated by CRF receptors. It appears that all of the basic factors necessary for LTDdepo induction, except CRF, were preserved in 3AP-treated rat cerebella and that the LTD deficit in these cerebella was due to the lack of endogenous CRF.

LTDdepo was restored only partially when 0.1 μM CRF was applied for only 15 min, starting 10 min before the LTDdepo-inducing combined stimulation; LTDdepo occurred in three of the nine cells tested (average LTD magnitude in the nine PCs, 12.5% ± 11.2% at 30 min after the onset of the combined stimulation). It was also observed that the effect of prolonged incubation with CRF was not blocked when α-h CRF (1.0 μM) was applied for only 15 min, starting 10 min before the onset of LTDdepo-inducing stimulation; LTDdepo occurred in two of the three cells tested. Thus, the restoration of LTD after replenishment of CRF in CF-deprived PCs and also the inhibition of the CRF-induced restoration in the presence of a CRF antagonist are slow processes. This is in contrast to the rapidity of the onset and recovery of the effect of CRF on AHP (Figure 2) and is presumed to be due to the slowness of the CRF-induced signal transduction process, such as that reported for phosphoinositide hydrolysis (Xiong et al., 1995) and PKC activation (Ishizuka et al., 1996).

Depolarization-Induced Ca^{2+} Signaling of PCs Was Unaffected by α-h CRF

The induction of LTD has been shown to involve a transient elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PCs caused by the activation of voltage-gated Ca^{2+} channels (Sakurai, 1990; Konnerth et al., 1992; Freeman et al., 1998). Therefore, the blockade of LTD by α-h CRF could be due to its depressant effect on depolarization-induced Ca^{2+} transients. To examine this possibility, we

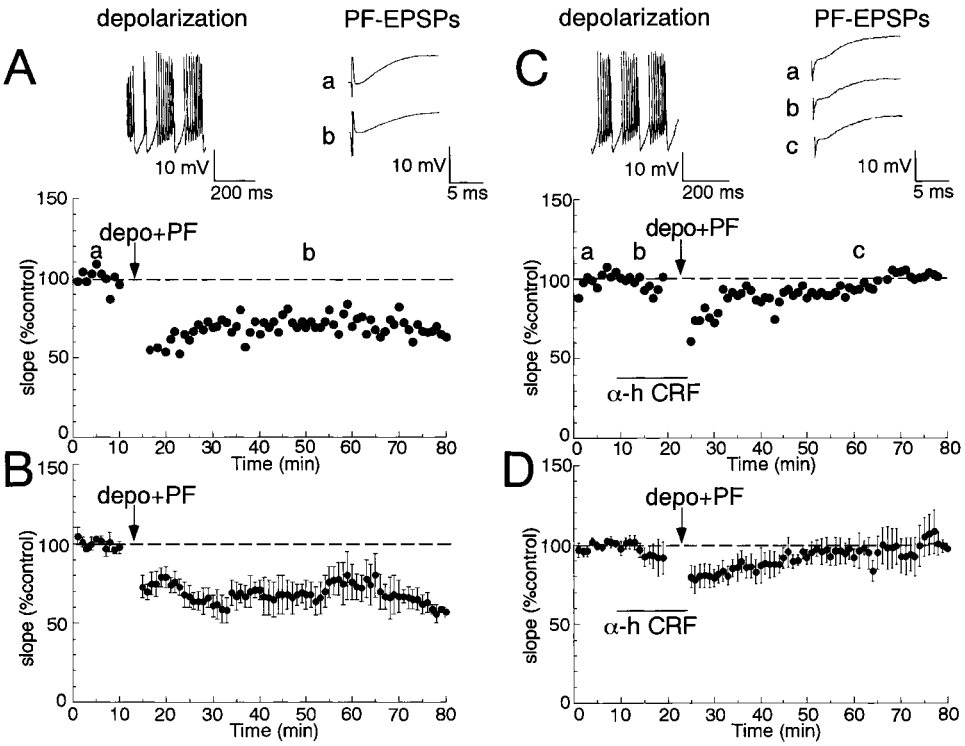

Figure 5. α-h CRF Blocks LTD Induction by a Combination of PF Stimulation and PC Depolarization (LTDdepo)

(A) PF stimulation combined (1 Hz for 5 min) with injections of depolarizing current pulses to PCs (0.5–0.8 nA, 200 ms) sufficient to evoke dendritic Ca^{2+} spikes (inset) induced LTD of PF-EPSP initial slopes. Sample records of PF-EPSPs were obtained (Aa) 5 min prior to and (Ab) 40 min following the initiation of the combined stimulation.

(B) Pooled data from seven experiments with a combination of PF stimulation and PC depolarization, performed as in (A). Error bars, SEM.

(C) Bath application of α-h CRF (1.0 μM) during the period indicated by the horizontal bar prevented induction of LTDdepo. Note that neither PF-EPSP nor depolarization-induced Ca^{2+} spiking was affected by α-h CRF. Subsequent combination of PF with PC depolarization resulted in a transient depression but not LTD of PF-EPSP initial slopes.

(D) Pooled data from 13 experiments combined PF stimulation and PC depolarization in the presence of α-h CRF, performed as in (C).

measured voltage-gated Ca^{2+} currents in standard and α-h CRF–containing perfusates using whole-cell voltage-clamp recordings. We used postnatal day 5 (P5) rats for these measurements, since at this developmental stage, PCs are devoid of extensive dendrites (Altman, 1972), such that the membrane voltage can be well controlled (Kano et al., 1995; Tempia et al., 1996). As exemplified in Figure 7A, bath application of α-h CRF had no depressant effect on voltage-gated Ca^{2+} currents in PCs. The peak amplitudes of the Ca^{2+} currents elicited by voltage steps to −10 mV were 2.31 ± 0.47 nA in the control and 2.32 ± 0.47 nA in the α-h CRF–containing perfusates (mean ± SEM, n = 7). To examine whether α-h CRF could have affected Ca^{2+} signaling processes after Ca^{2+} entry through the voltage-gated channels, we directly measured the depolarization-induced Ca^{2+} transients in the PCs of P20 rats. PCs were whole-cell voltage clamped with a patch-pipette containing a Ca^{2+} indicator dye, fura 2 (200 μM), and the $[Ca^{2+}]_i$ was measured with a single detector photomultiplier system. The basal $[Ca^{2+}]_i$ levels prior to depolarization were 50.7 ± 12.6 nM in the standard and 42.6 ± 11.2 nM in the α-h CRF–containing perfusates (mean ± SEM, n = 6). The peak Ca^{2+} transients induced by depolarizing pulses (from −60 mV to 0 mV; pulse duration, 200 ms) were 422.5 ± 160.6 nM in the standard and 468.8 ± 179.5 nM in the α-h CRF–containing perfusates, respectively (mean ± SEM, n = 6; Figure 7B, arrow). Thus, neither the basal $[Ca^{2+}]_i$ levels nor the Ca^{2+} transients were affected by α-h CRF. These results strongly suggest that the blockade of LTD by α-h CRF is not due to its effect on depolarization-induced Ca^{2+} signaling in PCs.

PKC Activation but Not cGMP Production Was Enhanced by CRF

Activation of PKC has also been shown to be required for LTD induction (Crepel and Jaillard, 1990; Linden and Connor, 1991; Hemart et al., 1995; De Zeeuw et al., 1998; Freeman et al., 1998). We therefore biochemically examined the effects of CRF and its antagonist on PKC activity. PKC activities in the particulate and cytosolic fractions of cerebellar slice homogenates were assayed separately, and the ratio of the particulate fraction to the sum of the particulate and cytosolic fractions was calculated to express the extent of membrane translocation of PKC. Membrane translocation of PKC occurs for productive phosphorylation of a membrane-bound protein and can therefore be considered as an index of PKC activation (Kraft and Anderson, 1983). As summarized in Table 1, membrane translocation of PKC was increased significantly after incubation with a PKC activator, 0.2 μM phorbol 12-myristate 13-acetate (PMA), for 15 min (from 30.4% to 47.8%) and also increased to a similar extent after incubation with 0.1 μM CRF for 30

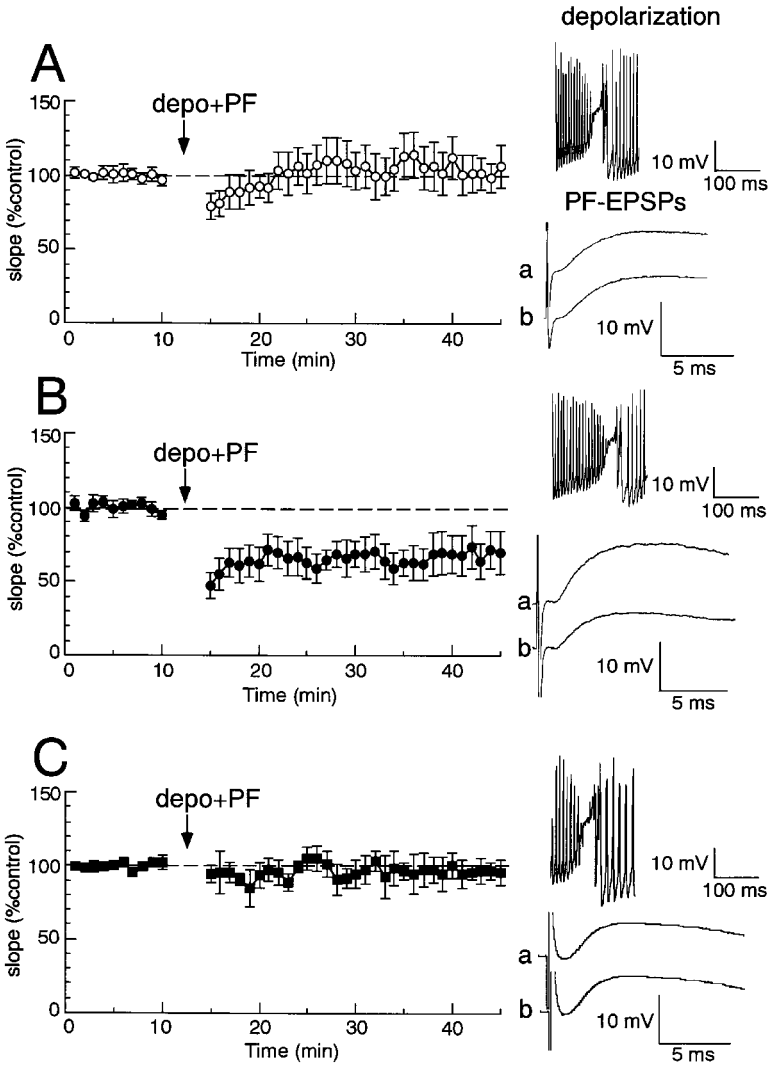

Figure 6. Chronic Destruction of CFs Abolishes LTDdepo, which Is Restored by CRF Replenishment

Pooled data obtained by a combination of PF stimulation (1 Hz for 5 min) and injections of depolarizing current pulses to PCs (0.5–0.8 nA, 200 ms) sufficient to evoke dendritic Ca^{2+} spikes in 3AP-treated, CF-deprived slices are illustrated as in Figure 5B. Sample records of PF-EPSPs in (A) through (C) were taken 5 min prior to (a) and 30 min after (b) initiation of combined stimulation.

(A) Eight experiments in normal perfusates.

(B) Seven experiments after 3 hr incubations with CRF (0.1 μM).

(C) Five experiments after incubation with both α-h CRF (1.0 μM) and CRF (0.1 μM). Note the failure of LTDdepo induction in (A) and (C) and its restoration in (B).

min (to 41.2%). Membrane translocation of PKC was slightly decreased (to 27.9%) in slices incubated with 1.0 μM α-h CRF, but the decrease was not statistically significant. In the slices obtained from 3AP-treated rats, membrane translocation of PKC significantly increased after incubation with 0.2 μM PMA for 15 min (from 35.6% to 50.2%) as well as after incubation with 0.1 μM CRF for 30 min (to 48.6%). The effect of CRF (0.1 μM) was blocked (35.9%) when α-h CRF (1.0 μM) was added to the perfusates. These observations indicate that PMA and CRF enhance membrane translocation of PKC to a similar extent in cerebellar neurons in both normal and CF-deprived slices. However, α-h CRF did not significantly affect the extent of membrane translocation of PKC.

NO formation, subsequent production of cGMP, and activation of plakoglobin (PKG) have also been shown to be required for LTD induction (Crepel and Krupa, 1988; Crepel and Jaillard, 1990; Ito and Karachot, 1990; Shibuki and Okada, 1991; Lev-Ram et al., 1995, 1997a, 1997b; Hartell, 1996b; Blond et al., 1997; Shibuki and Kimura, 1997). We therefore estimated biochemically the effect of CRF and its antagonist on cGMP production. The basal cGMP content in cerebellar slices was 25.2 ± 0.8 (mean ± SEM, n = 3) and 28.2 ± 1.5 (n = 7) pmol/mg protein in the absence and presence, respectively, of 0.1 mM 1-methyl-3 isobutylmethyl-xanthine (IBMX), a phosphodiesterase inhibitor, which is consistent with previously reported results (Okada, 1992). Incubation with either CRF or α-h CRF for 30 min did not affect the basal cGMP level measured in the presence of 0.1 mM IBMX (0.1 μM CRF, 31.8 ± 6.2 pmol/mg protein, n = 4; 1.0 μM α-h CRF, 29.1 ± 2.2 pmol/mg protein, n = 5). The levels of cGMP in cerebellar slices increased markedly in the presence of sodium nitroprusside (SNP) (0.1 μM SNP, 120.9 ± 10.7, n = 5). This increase was not significantly affected by either 0.5 μM CRF (112.5 ± 52.0, n = 3) or 1.0 μM α-h CRF (157.2 ± 68.9, n = 3). These results suggest that CRF does not affect the intracellular regulatory mechanisms of cGMP concentration.

Discussion

Requirement of CRF for LTD Induction

The present study has established that the presence of CRF is critical for LTD induction, based on two lines of evidence; first, both LTDcnj and LTDdepo are abolished

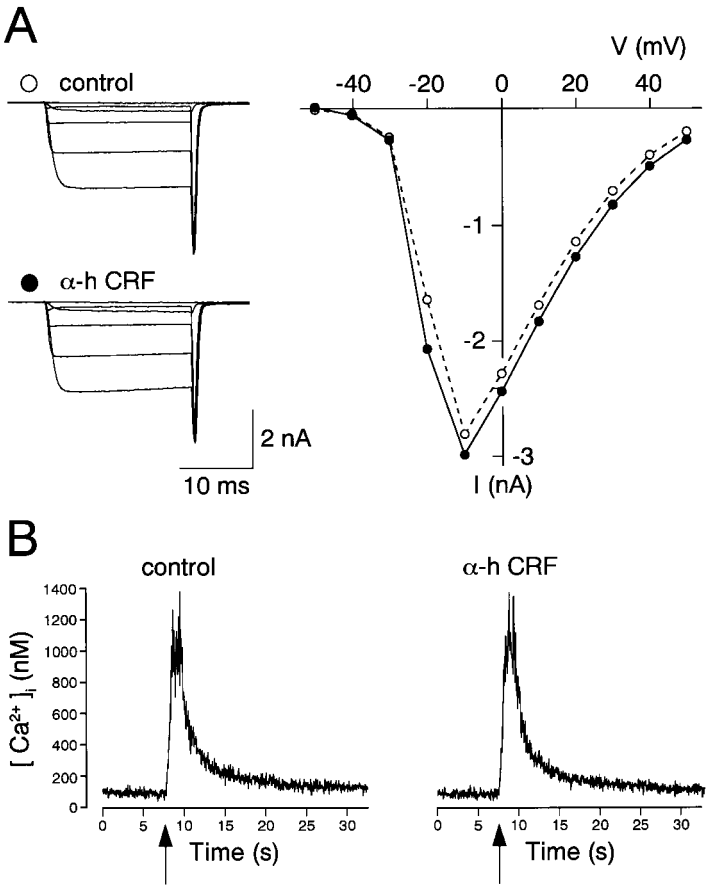

Figure 7. α-h CRF Has No Depressant Effect on Voltage-Gated Ca^{2+} Currents or Depolarization-Induced Ca^{2+} Transients in PCs

(A, left) Whole-cell membrane currents elicited with 20 ms voltage steps from a holding potential of −60 mV to various test potentials of −40 mV, 0 mV, +10 mV, +20 mV, and +30 mV. Records were obtained from a 5-day-old rat before (upper) and after (lower) bath application of α-h CRF (1.0 μM).

(A, right) Current–voltage relations for peak Ca^{2+} currents obtained from the same PC before (open circles) and after (closed circles) bath application of α-h CRF (1.0 μM).

(B) Ca^{2+} transients of a PC from a 21-day-old rat in response to depolarizing pulses (from 60 to 0 mV; pulse duration, 200 ms). Recordings were obtained before (left) and after (right) bath application of α-h CRF (1.0 μM).

by CRF receptor antagonists (Figures 3 and 5), and second, LTDdepo was abolished in the cerebella of rats whose CFs were destroyed by 3AP (Figure 6A). Because α-h CRF and astressin blocked not only LTDcnj (Figure 3) but also LTDdepo, which is induced without stimulation of CFs (Figure 5), it is likely that the ambient CRF present in cerebellar slices, probably released spontaneously from CFs, is sufficient for LTD induction. Since CRF secretion has been reported from unstimulated rat cerebellar slices (G. A. Bishop et al., 1997, Soc. Neurosci., abstract), it is likely that cerebellar tissues contain a certain basal level of CRF. This basal level of CRF, however, is probably low, around 0.1 μM or even less, too low to reduce the AHP amplitude, since α-h CRF did not affect the latter (see Results); if the basal level had been high, 0.5–1.0 μM, enough to reduce the AHP amplitude, α-h CRF should have increased AHP (cf. Figure 2A). The addition of CRF at 0.1 μM was sufficient to restore LTD in CF-deprived cerebellar slices (Figure 6) and to increase membrane translocation of PKC (Table 1). Therefore, AHP amplitude is not a sufficiently sensitive indicator to detect the postulated ambient CRF released from CFs. Another possibility that must be considered is that K^+ ions driven out of PCs during membrane depolarizations depolarize CF terminals, leading to the release of CRF. This is, however, unlikely, since LTD can also be induced by Ca^{2+} ions photolytically released inside PCs without CF stimulation or PC depolarization in cerebellar slices (Lev-Ram et al., 1997a).

Our results suggest that the CRF receptor cascade is not involved in the maintenance but does play a role in the induction of LTD, because α-h CRF had no effect on the late phase (Figures 4A and 4B), which in cultured PCs is protein synthesis–dependent (Linden, 1996). Since the rescue of LTD by CRF in CF-deprived PCs is blocked by CRF antagonists (Figure 6), it is likely that the effect of CRF is mediated by specific CRF receptors present in the PCs.

Table 1. PKC Activity in Cerebellar Slices

PKC Translocation (%)	Control	PMA	CRF	α-h CRF	CRF+ α-h CRF
Normal slices (n)	30.4 ± 2.3 (4)	47.8 ± 3.8** (3)	41.2 ± 2.4** (5)	27.9 ± 2.3 (3)	—
CF-deprived slices from 3AP-treated rats (n)	35.6 ± 1.8 (4)	50.2 ± 4.5** (4)	48.6 ± 2.4** (4)	—	35.9 ± 2.0 (3)

After 15 min incubation in normal medium containing 0.2 μM phorbol 12-myristate 13-acetate (PMA). CRF, 0.1 μM for 30 min. α-h CRF, 1 μM for 15 min. CRF + α-h CRF, simultaneous application of 0.1 μM CRF and 1.0 μM α-h CRF for 30 min. Numerical figures, mean ± SEM. In parentheses, number of experiments performed. Double asterisk, $p < 0.01$ compared with control, unpaired t test.

The results of this study, demonstrating that CRF antagonists blocked LTD, may be at variance with the finding that a combination of glutamate and depolarization pulses induces LTD in cultured PCs (Linden et al., 1991; Hirano et al., 1994), since cultured PCs are devoid of CFs, and presumably consequently, CRF. However, the presence of CRF receptors in cultured PCs is apparent, because their AHP amplitude is readily decreased by CRF (Fox and Gruol, 1993). It may be worthwhile examining if ambient CRF is present in cellular element(s) other than CFs in cell cultures derived from very young rats. If no CRF is present in the tissue cultures, it should be considered that signal transduction processes underlying LTD differ in certain respects between cerebellar tissues and tissue cultures. This is probably the case, because a marked difference is already known to exist; NO is required for LTD induction in cerebellar slices (Lev-Ram et al., 1997b) but not in cultured PCs (Linden et al., 1995). Since six pulses of glutamate application, combined with depolarization, are sufficient to induce LTD in cultured PCs (Linden, 1995), while 300 pulses of conjunctive PF and CF stimuli are necessary to induce LTD in cerebellar slices (Karachot et al., 1994), it is possible that signal transduction processes leading to LTD are in a readily activated state in cultured PCs, whereas a synergistic effect of CRF is required for sufficient activation of the signal transduction processes in PCs in cerebellar slices.

Negative Evidence for the Involvement of Glutamate Receptors, Ca^{2+} Signaling, and the NO–cGMP Pathway in the Induction of LTD

Ionotropic AMPA–type glutamate receptors that mediate excitatory transmission at PF synapses to PCs (Llinano et al., 1991) have been reported to be activated for LTD induction (Kano and Kato, 1987, 1988; Linden et al., 1991; Hemart et al., 1995). However, since CRF antagonists did not affect PF-EPSPs, except in the case of LTD, AMPA receptors in PF synapses are unlikely to be the site of action of CRF for LTD induction. mGluR1s that are colocalized with AMPA receptors at dendritic spines postsynaptic to PF terminals (Baude et al., 1993; Nusser et al., 1994) are also probably activated for LTD induction (Aiba et al., 1994; Conquet et al., 1994; Hartell, 1994; Shigemoto et al., 1994). However, since CRF antagonists did not affect mGluR1-mediated slow EPSPs in PCs (Figure 2B), these receptors can also be excluded as the site of interference between CRF receptor activation and LTD induction.

Another requirement for LTD induction is a transient elevation of $[Ca^{2+}]_i$ in PC dendrites, mediated by the activation of voltage-gated Ca^{2+} channels (Sakurai, 1990; Konnerth et al., 1992). Since depolarization-induced Ca^{2+} signaling in PCs was not affected by CRF antagonists (Figure 7), interference by CRF of Ca^{2+} signaling such as was reported in corticotrophs (Guerineau et al., 1991) is unlikely to occur in PCs. Ca^{2+} signaling can thus also be excluded as a site of action of CRF for LTD induction.

It is of interest to discuss here the source of Ca^{2+} signaling in LTD induction. This has been considered to be caused by CF input, since fluorometric Ca^{2+} measurements have demonstrated prominent Ca^{2+} elevation in response to CF activation (Knöpfel et al., 1990; Konnerth et al., 1992; Miyakawa et al., 1992). However, it has recently been reported that under certain conditions, PF stimulation also causes significant Ca^{2+} elevation (Eilers et al., 1995; Hartell, 1996a); in particular, strong and repetitive PF stimulation that yields EPSPs larger than 10 mV at peak induced significant Ca^{2+} influx into PC dendrites and caused persistent depression of PF-EPSPs (Hartell, 1996a). These experiments were performed on PC somata under whole-cell recording conditions in thin (200 μm) cerebellar slices obtained from young (P14–P21) rats, while in the present study, we recorded PF-EPSPs from proximal dendrites of PCs in 400 μm thick slices obtained from rats aged 42–49 days. We stimulated PFs at a low frequency of 0.2 Hz and adjusted the stimulus intensity so as to yield PF-EPSPs of 8–15 mV in amplitude. Since PF-EPSPs are much larger when recorded in proximal dendrites than in the somata (Sakurai, 1987), the PF stimuli adopted in the present study probably did not induce significant Ca^{2+} influx, if any, into PC dendrites. In fact, as shown in Figures 1C and 1D, repetitive PF stimulation at 1 Hz for 5 min (300 stimuli) did not induce depression and instead caused a slight potentiation of PF-EPSPs. This is consistent with previous reports using similar experimental protocols (Sakurai, 1987, 1990; Karachot et al., 1994). Thus, under our recording conditions, the Ca^{2+} influx into PCs that was necessary for LTD induction was caused by CF inputs (LTDcnj) or the direct depolarization of PCs (LTDdepo).

LTD induction has been reported to require NO, which is yielded by activation of NO synthase in PFs and which in turn activates guanylate cyclase in PCs, leading to the generation of cGMP and activation of PKG (Ariano et al., 1982; Shibuki and Okada, 1991; Lev-Ram et al., 1995; Hartell, 1996b; Shibuki and Kimura, 1997). Since, however, the levels of cGMP in cerebellar slices, regardless of the absence or presence of a NO donor, were not significantly affected by either CRF or α-h CRF in the present experiment, the NO–cGMP pathway can be excluded from consideration as the site of action of CRF in LTD interaction.

Since type 1 CRF receptors are reported to be coupled with Gs proteins (Battaglia et al., 1987), there is a possibility that CRF activates adenylate cyclase via Gs proteins, induces the production of cAMP, and thereby activates PKA; however, there is no evidence suggesting the involvement of PKA in LTD induction (Ito and Karachot, 1992; Storm et al., 1998).

Possible Involvement of PKC

PKC is activated downstream of the activation of mGluR, and its activation has been reported to be required for LTD induction (Crepel and Krupa, 1988; Crepel and Jaillard, 1990; Linden and Connor, 1991; De Zeeuw et al., 1998). It has been reported that CRF activates PKC in pituitary cells and A-431 cells (Kiang et al., 1994; Ishizuka et al., 1996). Indeed, we observed CRF-enhanced membrane translocation of PKC in control cerebellar slices (Table 1). In CF-deprived cerebellar slices, membrane translocation of PKC was similarly enhanced by CRF, and this enhancement was blocked by α-h CRF. These

data must be evaluated with caution, because the present biochemical measurements do not distinguish between the different cell types contained in cerebellar slices. Nevertheless, under the assumption that the measurements reflect changes of PKC activity in PCs, at least to a significant extent, the data in Table 1 suggest that the restoration of LTD induction by CRF in CF-deprived PCs is due to enhanced PKC activation caused via CRF receptors.

It is noted, however, that membrane translocation of PKC was not significantly affected by α-h CRF in control cerebellar slices or by CF deprivation in 3AP-treated slices. Probably, the basal level of PKC activity is largely maintained by drives other than CRF receptor–mediated mechanisms. The effect of CRF antagonists in blocking LTD induction is therefore not due to a lowered activation level of PKC but possibly to insufficient elevation of PKC activity during conjunctive CF and PF stimulation. The latter possibility is difficult to examine with the present biochemical measurements, because conjunctive stimulation involves only a small fraction of a cerebellar slice. Since PF signals may enhance PKC activity via activation of mGluR1 and subsequent production of diacylglycerol (DAG), there is a possiblity that CRF facilitates the mGluR1–DAG pathway, such that both the presence of CRF receptor antagonists and deprivation of CRF lead to an insufficient elevation of PKC activity during conjunctive stimulation. Experimental testing of this possibility will be the focus of another study.

In parallel with DAG production and subsequent PKC activation, phosphoinositide hydrolysis is promoted by activation of mGluR and in turn leads to release of Ca^{2+}, which is required for LTD induction (Inoue et al., 1998), from intracellular stores. Since phosphoinositide hydrolysis is activated via type-1 human and mouse CRF receptors in cDNA-transfected COS-7 cells (Xiong et al., 1995), it is possible that CRF receptor activation leads to LTD induction by modulating phosphoinositide hydrolysis. Presently, however, no data are available to substantiate this possibility.

Protein tyrosine kinases (PTKs) have also been reported to be required for LTD induction, because PTK inhibitors abolish LTDdepo (Boxall et al., 1996). Since depression of PF-EPSPs induced by a PKC activator was also prevented by a PTK inhibitor, it has been suggested that PKC activates PTKs, which in turn modify the functions of receptors (Boxall and Lancaster, 1998). However, modification of AMPA receptors by PTKs has not yet been proven, and the possibilities that remain to be examined are that PKC and PTKs work in parallel, converging further downstream rather than in series, or even that PKC activity is affected by PTKs (Umemori et al., 1997). Presently, no data are available for or against the involvement of PTKs in CRF–LTD interaction.

To summarize, PKC activation is suggested as a possible mechanism of CRF–LTD interaction, whereas the involvement of glutamate receptors, Ca^{2+} signaling, the NO–cGMP–PKG pathway, and PKA is excluded. Phosphoinositide hydrolysis and PTKs are still possibilities that remain to be examined. The possibility that CRF receptor activation involves an as yet unknown signal transduction process for LTD induction must also be borne in mind.

Experimental Procedures

Preparation of Cerebellar Slices

Male Wistar rats of three different age groups (P4–P6, P20–P25, P42–P49) were used. They were decapitated under ether anesthesia, and the cerebellar vermis was dissected out. Parasagittal cerebellar slices of either 200 or 400 μm thickness were prepared with a microslicer (Dosaka). The slices were incubated at room temperature in a perfusion medium with the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 7; H_2O, 1.2; and glucose, 11, equilibrated with 95% O_2 + 5% CO_2 gas (pH 7.4). One slice was transferred to a submerged recording chamber, maintained at 32°C, and perfused with perfusion medium containing 20 μM picrotoxin at the rate of 2 ml/min. α-h CRF was dissolved in DMSO (final concentration, 0.002%); α-h CRF and CRF (human, rat) were purchased from Sigma. Astressin was a generous gift from Dr. Toshihiro Imaki (Tokyo Women's Medical University).

In some experiments, CFs were denervated pharmacologically with a combination of 3AP, harmaline, and niacinamide (Llinás et al., 1975). The rats (21 days old) were injected with 3AP intraperitoneally (75 mg/kg). Harmaline (15 mg/kg) and niacinamide (300 mg/kg) were also administered intraperitoneally 3 and 4.5 hr, respectively, after the 3AP injection. Parasagittal slices were prepared from these rats 60–90 days after the 3AP treatment. For the results shown in Figures 6A and 6B, the solution containing CRF or the control perfusion medium was blindly applied to the slices.

Stimulation and Intradendritic Recording from PCs

Bipolar stimulation electrodes were prepared from two twisted platinum iridium wires separated, except at the very tip (50 μm outer diameter). The electrodes were gently placed on the pial surface and white matter to stimulate the PFs and CFs, respectively. A glass microelectrode filled with 3 M KCl solution and having a resistance of 60–80 MΩ was inserted into the PC somata or dendritic shaft in the middle of the molecular layer. Recordings were obtained with a standard intracellular recording amplifier with current injection and bridge balancing capabilities (Nihon Kohden MEZ8301). PFs were stimulated at 0.2 Hz, and five consecutive sweeps were averaged and stored online with patch-clamp software (Axon Instruments). For directly evoking Ca^{2+} spikes, depolarizing current pulses of 0.5–0.8 nA (duration, 200 ms) were applied through the microelectrode. Only those data obtained when the resting potential and membrane resistance remained constant were considered for later analysis.

Measurement of $[Ca^{2+}]_i$ and Voltage-Gated Ca^{2+} Currents

PCs in cerebellar slices (200 μm thick) were visualized through the 40× water immersion objective of an upright microscope (Olympus BH-2). Whole-cell patch-clamp recording methods were used to record membrane currents (Edwards et al., 1989) using an Axopatch 1D amplifier (Axon). Only data from cells whose access resistance did not exceed 10–15 MΩ, even during prolonged recording periods, were considered for further analysis. Fura 2 (200 μM, Dojin, Japan) was added to the pipette solution containing (in mM): CsCl, 60; CsD-gluconate, 30; TEA-Cl, 20; $MgCl_2$, 4; Na-ATP, 4; Na-GTP, 0.4; and HEPES, 30 (pH 7.3, adjusted with CsOH). Fluorescence was measured with a single detector photomultiplier system (Olympus, OSP-3).

For measuring voltage-gated Ca^{2+} channel currents in the PCs of 4- to 6-day-old rats, extracellular Ca^{2+} ($CaCl_2$, 2 mM) was replaced with $Ba2^{+}$ ($BaCl_2$, 5 mM) to prevent Ca^{2+}-induced inactivation of Ca^{2+} channel currents. Tetrodotoxin (TTX; 0.5 μM) and tetraethyl ammonium (TEA; 75 mM) were included in the external solution to prevent Na^{+} and K^{+} currents, and the concentration of NaCl was reduced to 50 mM to keep the osmolarity constant.

Biochemical Assay

For measuring PKC activity, parasagittal slices of the vermis (400 μm thick) were prepared from Wistar or 3AP treated rats and preincubated in the standard medium for 1 hr at 33°C before being immersed in perfusates containing various reagents. Slices were homogenized in 1 ml of buffer A (25 mM Tris-HCl [pH 7.5], 50 mM

2-mercaptoethanol, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.005% leupepsin, and 0.25 M sucrose). The homogenate was centrifuged at 100,000 × g for 1 hr. PKC activity bound to the particulate fraction was extracted by resuspending the pellet in 1 ml of buffer A containing 1% Triton X-100 followed by gentle stirring at 4°C for 1 hr. The extract was collected by centrifugation as described above. The cytosol and extract were loaded onto DE52 (Whatman) columns preequilibrated with buffer B (25 mM Tris-HCl [pH 7.5], 50 mM 2-mercaptoethanol, 2 mM EGTA, and 0.002% leupepsin) and then washed with it. PKC was eluted with buffer B containing 130 mM NaCl, and its activity in 40 μl of the eluant was assayed with 0.2 mg/ml of histone III-S, as the phosphate acceptor; 20 μM ATP; and 1.5 MBq of [γ-^{32}P]ATP (Okada, 1996).

For cGMP assay, homogenates were similarly obtained from the cerebellar slices. The method used for cGMP assay has been described in detail previously (Okada, 1992). The cGMP content is expressed in picomoles per milligram of protein, in mean ± SEM values.

Calbindin D28K Staining

Coronal sections of the cerebellum (10 μm) were prepared at 40 μm intervals. Calbindin D28K immunohistochemistry was performed as described by Celio (1990). The density of PCs was obtained from each section as the cell number per 0.1 mm^2 of lobules 3 and 4 with the MicroComputer Imaging Device (Imaging Research, Ontario, Canada).

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